Laboratory Transmission of Japanese Encephalitis and West Nile Viruses by Molestus Form of *Culex pipiens* (Diptera: Culicidae) Collected in Uzbekistan in 2004

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ABSTRACT   We evaluated the molestus form of *Culex pipiens pipiens* (L.) (hereafter referred to as "molestus") captured near Tashkent, Uzbekistan, for their ability to transmit Japanese encephalitis (family *Flaviviridae*, genus *Flavivirus*, JEV) and West Nile (family *Flaviviridae*, genus *Flavivirus*, WNV) viruses under laboratory conditions. These molestus were highly competent laboratory vectors of WNV, with infection and dissemination rates of 96 and 81%, respectively. Approximately 75% of female molestus that fed after development of a disseminated infection transmitted virus by bite. Therefore, ≈60% of those molestus taking a second bloodmeal between 16 and 25 d after an infectious bloodmeal would be expected to transmit WNV by bite. In contrast, these molestus were less efficient vectors of JEV, with infection and dissemination rates of 51 and 25%, respectively. In addition, only 33% of individuals with a disseminated infection transmitted JEV by bite, indicating a significant salivary gland barrier. Therefore, only ≈8% of orally exposed individuals would be expected to transmit JEV by bite if they took a second bloodmeal 16–25 d later. These data indicate that the molestus form of *Cx. p. pipiens* should be considered a potentially important vector of WNV in Uzbekistan and may become involved in the transmission of JEV, should this virus be introduced into Uzbekistan.

KEY WORDS   transmission, mosquitoes, Uzbekistan, Japanese encephalitis, West Nile

West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV), a member of the Japanese encephalitis (family *Flaviviridae*, genus *Flavivirus*, JEV) serogroup is known to occur over a wide geographic area. It has been associated with illness in humans in much of Africa, southern/central Europe, the Middle East, Australia (as Kunjin virus), Asia (east to the Indian highlands), and more recently the Americas (Hayes 1989, Lvov et al. 2004, Mackenzie et al. 2004), with major outbreaks occurring in North America, the Middle East, eastern Europe, and southern Russia in the last decade (CDC 1999, Lanciotti et al. 1999, Savage et al. 1999, Lvov et al. 2004, Mackenzie et al. 2004). Although this virus has been isolated from numerous species of mosquitoes (Hayes 1989, Hayes et al. 1982, Hubalek and Halouzka 1999, CDC 2005), most have been made from members of the subgenus *Culex* (*Culex*). Many mosquito species are competent vectors for WNV in the laboratory (Hubalek and

Halouzka 1999, Turell et al. 2005). The prototype of this serogroup, JEV, is enzootic in Asia from western Nepal to Korea and Japan and has been responsible for outbreaks of encephalitis in humans, with thousands of cases being reported each year (Burke and Leake 1988, Vaughn and Hoke 1992, Sohn 2000, Mackenzie et al. 2004).

Outbreaks of illness associated with WNV have occurred in Uzbekistan, causing both febrile illness and meningoencephalitis (Meliev et al. 1980, Meliev and Shermuhamedova 1984). In addition, seroprevalence against WNV have ranged from 2.2 to 9.4% in seven regions of Uzbekistan (Meliev et al. 1980, Brjantseva et al. 1993). Although members of the genus *Culex* have been associated with the enzootic transmission of WNV (Hayes 1989, Hubalek and Halouzka 1999), mosquitoes from Uzbekistan have never been evaluated for their ability to transmit either WNV or the closely related JEV. Therefore, we investigated the potential for *Culex* mosquitoes, captured near Tashkent, Uzbekistan, to transmit these viruses.

Materials and Methods

Mosquitoes. Adult *Culex* mosquitoes were collected in dry ice-baited miniature light traps (John W. Hock Co., Gainesville, FL) in July 2004. Traps were operated from sunset until dawn in three locations in the vi-

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14. ABSTRACT
We evaluated the molestus form of Culex pipiens pipiens (L.) (hereafter referred to as "molestus") captured near Tashkent, Uzbekistan, for their ability to transmit Japanese encephalitis (family Flaviviridae, genus Flavivirus, JEV) and West Nile (family Flaviviridae, genus Flavivirus, WNV) viruses under laboratory conditions. These molestus were highly competent laboratory vectors of WNV, with infection and dissemination rates of 96 and 81%, respectively. Approximately 75% of female molestus that fed after development of a disseminated infection transmitted virus by bite. Therefore, approximately 60% of those molestus taking a second bloodmeal between 16 and 25 d after an infectious bloodmeal would be expected to transmit WNV by bite. In contrast, these molestus were less efficient vectors of JEV, with infection and dissemination rates of 51 and 25%, respectively. In addition, only 33% of individuals with a disseminated infection transmitted JEV by bite, indicating a significant salivary gland barrier. Therefore, only approximately 8% of orally exposed individuals would be expected to transmit JEV by bite if they took a second bloodmeal 16-25 d later. These data indicate that the molestus form of Cx. p. pipiens should be considered a potentially important vector of WNV in Uzbekistan and may become involved in the transmission of JEV, should this virus be introduced into Uzbekistan.

15. SUBJECT TERMS
Japanese B encephalitis virus, West Nile virus, mosquito vectors, Culex pipiens, Uzbekistan, virus transmission

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cinity of Tashkent, Uzbekistan (41° 16′ N, 69° 13′ E). One of the traps, which captured the most mosquitoes, was located ≈10 m from a large, cement underground water storage tank, and there were adult *Culex* mosquitoes in this storage tank. Captured mosquitoes were transported to the United States Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD, where they were provided apple slices as a carbohydrate source, held at 26°C, and allowed to feed on uninfected, anesthetized hamsters to stimulate egg production. Eggs collected from these females were allowed to hatch, and the resulting larvae were provided ground catfish chow (AquaMax Pond Plus 3000, Purina Mills, Inc., St. Louis, MO) and reared at 26°C. These F_1 adults were used to evaluate the potential for these mosquitoes to become infected with and transmit WNV and JEV.

The colony we established for use in these studies is both autogenous and stenogamous and therefore seems to be the autogenous form of *Culex pipiens pipiens* (L.) described by Fonseca et al. (2004), which for brevity we will call "molestus." Specimens from this colony were tested using the same eight microsatellite loci as was done in Fonseca et al. (2004) and found to be *Cx. p. pipiens* variety molestus (D. Fonseca, unpublished data). Voucher specimens were deposited at the National Museum of Natural History, Smithsonian Institution, Washington, DC. This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Virus and Assays. We used a strain of JEV (ROK-2.0028) previously isolated from *Culex tritaeniorhynchus* Giles captured near Camp Greaves in 2000 (Turell et al. 2003) and passaged twice in African green monkey kidney (VERO) cells before use in this study. We also used a strain of WNV (crow 397–99) from the brain of a crow that died in the Bronx, NY, in September 1999 (Turell et al. 2000). This strain had been passaged once in VERO cells before use in this study.

To determine their infection status, specimens were serially diluted in diluent (10% heat-inactivated fetal bovine serum in medium 199 with Earle's salts, $NaHCO_3$, and antibiotics) and tested for the presence of virus on Vero cell monolayers by plaque assay. Procedures for plaque assay were similar to those described by Gargan et al. (1983) except that the overlay containing neutral red was added 2 d (WNV) or 4 d (JEV) after the initial assay. Plaques were enumerated the following day.

Viremia Profile Studies. Preliminary studies determined viremia profiles for JEV in young leghorn chickens (*Gallus gallus*). One- to 2-d-old chickens were inoculated subcutaneously with 0.1 ml of a suspension containing ≈10^4 plaque-forming units (PFU) of JEV.

These chickens were bled daily from the jugular vein (0.1 ml of blood into 0.9 ml of heparinized diluent), and the blood suspensions were frozen at −70°C until tested for virus by plaque assay. Viremia profiles for WNV-infected chickens indicated that viremias ≈10^7 PFU/ml occurred 2 to 3 d after infection of 1-d-old chickens (Turell et al. 2000).

Vector Competence Studies. Uninfected mosquitoes were transferred to a biological safety level-3 laboratory with HEPA-filtered exhaust air, treated sewage, and a 100% clothing change and allowed to feed on 2- to 4-d-old leghorn chickens that had been inoculated with 10^4 PFU of JEV or WNV 2 to 3 d earlier. Immediately after the mosquitoes fed, 0.1 ml of blood was obtained from the jugular vein of each chicken and handled as describe above to determine the viremias at the time of mosquito feeding. After exposure to the viremic chickens, fully engorged mosquitoes were transferred to 3.8-liter screen-topped cardboard cages and held at 26°C at a photoperiod of 16:8 (L:D) h. After an incubation period of ≥16 d, some of the mosquitoes were allowed to refeed on 1- to 2-d-old chickens either individually or in small groups to determine whether they could transmit virus by bite. Immediately after the transmission attempt, the mosquitoes were killed by freezing, their feeding status was determined, and their legs and bodies were triturated separately in 1 ml of diluent. Infection was determined by recovery of virus from the mosquito tissue suspension. If virus was recovered from its body, but not its legs, the mosquito was considered to have a nondisseminated infection limited to its midgut. In contrast, if virus was recovered from both the body and leg suspensions, the mosquito was considered to have a disseminated infection (Turell et al. 1984). We defined the infection and dissemination rates as the percentages of mosquitoes tested that contained virus in their body or legs, respectively. Chickens used in the transmission attempts were bled from the jugular vein 1 or 2 d (for chickens exposed to WN and JEV, respectively) after mosquito feeding, and the blood handled as described previously. Recovery of virus from this blood indicated transmission. Some of the mosquitoes were also tested for their ability to transmit virus to diluent in a capillary tube (Aitken 1977). Briefly, mosquitoes were chilled in a glass container in wet ice. Their legs were removed and triturated for virus testing, their wings removed, and their bodies placed on their sides on sticky tape. A glass capillary tube containing ≈10 μl of diluent (fortified to 50% heat-inactivated fetal bovine serum) was placed so that the mosquito's proboscis was inserted into the diluent. Thirty minutes later, the diluent was expressed into 500 μl of diluent, and the mosquito's body was triturated for virus testing. The diluent, containing the expressed saliva, was tested on six-well plates in triplicate for the presence of virus. Infection and dissemination rates were compared by either chi-square or Fisher exact tests at the 95% confidence level.

Table 1. Viremia titers in 1-d-old chickens inoculated with Japanese encephalitis virus

	Day after infection		
1	2	3	4
4.3 ± 0.26 (3)	4.8 ± 0.54 (15)	4.9 ± 0.51 (9)	4.5 ± 1.12 (6)

Mean $\pm$ SD of $\log_{10}$ PFU/1 ml (number of chickens tested).

Results and Discussion

Viremias in chickens inoculated with JEV were detectable 24 h after inoculation and generally peaked at 2 to 3 d after inoculation (Table 1). Based on these data, we allowed the mosquitoes to feed on chickens 2 or 3 d after inoculation.

Viremias in the three chickens used to expose mosquitoes to WNV ranged from $10^{6.3}$ to $10^{7.0}$ PFU/ml of blood, whereas those in the three chickens used to expose mosquitoes to JEV ranged from $10^{4.5}$ to $10^{5.4}$ PFU/ml of blood. For each of the two viruses tested, infection and dissemination rates were similar over the dose ranges tested. Therefore, data for the mosquitoes exposed to each of the three chickens were combined for further analysis for both viruses.

The molestus from Uzbekistan were highly susceptible to infection with WNV (Table 2). Overall, 96% of the mosquitoes became infected, and 81% (84% of infected mosquitoes) developed a disseminated infection by 16 d after oral exposure, the first date sampled. Infection and dissemination rates were similar at all time intervals tested, 16–25 d after the infectious bloodmeal. Only 13 individual mosquitoes took a second blood meal, and six (46%) of them transmitted WNV by bite. This included six (75%) of the eight mosquitoes with a disseminated infection. Because 81% of the mosquitoes had developed a disseminated infection, we would expect that 60% (0.81 dissemination rate $\times$ 0.75 transmission rate) of this population of the molestus form of *Cx. pipiens* would transmit WNV $\geq$16 d after feeding on an animal with a viremia $\geq 10^{6.5}$ PFU/ml.

Although infection rates in the molestus from Uzbekistan were similar to those for *Cx. pipiens* cap-

Table 2. Infection, dissemination, and transmission rates for *Cx. p. pipiens* var. *molestus* orally exposed to West Nile virus

	Day of extrinsic incubation			
	16–17	20	25	Total
No. tested	50	53	37	140
Infection rate[a]	100	96	89	96
Dissemination rate[b]	78	87	76	81
Transmission rate (n)[c]	n.t.	47 (15)	n.t.	47 (15)
Transmission rate dissem. (n)[d]	n.t.	70 (10)	n.t.	70 (10)

n.t., not tested
[a] Percentage of mosquitoes containing virus in their bodies.
[b] Percentage of mosquitoes containing virus in their legs.
[c] Percentage of mosquitoes that refed that transmitted virus (number refed).
[d] Percentage of mosquitoes with a disseminated infection that refed that transmitted virus (number of mosquitoes with a disseminated infection that refed).

Table 3. Infection, dissemination, and transmission rates for *Cx. p. pipiens* var. *molestus* orally exposed to Japanese encephalitis virus

	Day of extrinsic incubation			
	16–17	19	25–27	Total
No. tested	50	53	39	142
Infection rate[a]	56	47	51	51
Dissemination rate[b]	26	25	26	25
Transmission rate (n)	n.t.	8 (24)[c]	8 (13)[c]	8 (37)[c]
Transmission rate dissem. (n)	n.t.	29 (7)[d]	50 (2)[d]	33 (9)[d]

n.t., not tested.
[a] Percentage of mosquitoes containing virus in their bodies.
[b] Percentage of mosquitoes containing virus in their legs.
[c] Percentage of mosquitoes that refed that transmitted virus (number refed). This included transmission by two of 24 mosquitoes to chickens on day 19 and one of 13 via the capillary method on day 27.
[d] Percentage of mosquitoes with a disseminated infection that refed that transmitted virus (number of mosquitoes with a disseminated infection that refed). This included transmission by two of seven mosquitoes to chickens on day 19 and one of two via the capillary method on day 27.

tured in New York state, dissemination of WNV was significantly more efficient ($\chi^2 = 74$, df $= 1$, $P < 0.001$) in the molestus form of *Cx. pipiens* from Uzbekistan (81% with a disseminated infection compared with 23% in those from New York; Turell et al. 2001). However, the mosquitoes from New York were tested after a shorter extrinsic incubation period, 14 d, compared with 16–25 d for the mosquitoes from Uzbekistan. In a study by Dohm et al. (2002), where mosquitoes were tested after a variety of incubation periods, the dissemination rate increased after 14-d extrinsic incubation and was $\approx$80% for those *Cx. pipiens* held for 16–25 d at 26°C. Therefore, the increased dissemination rates observed in our study may have been because of an increased incubation period rather than molestus from Uzbekistan being more susceptible to developing a disseminated WNV infection.

Although this population of the molestus form of *Cx. pipiens* was highly susceptible to WNV, it was only moderately susceptible to infection with JEV. Overall, 51% of the molestus became infected and 25% (49% of infected mosquitoes) developed a disseminated infection by 16 d after oral exposure, the first date sampled (Table 3). Infection and dissemination rates were similar at all time intervals tested, 16–27 d after the infectious bloodmeal. Twenty-four mosquitoes took a second bloodmeal, and two (8%) transmitted JEV by bite. Saliva was collected from 13 additional mosquitoes, and one (8%) of them also transmitted JEV. Therefore, three (8%) of 37 mosquitoes transmitted JEV after oral exposure. This included three (33%) of nine mosquitoes with a disseminated infection. Because 25% of the mosquitoes had developed a disseminated infection, we would expect that 8% (0.25 $\times$ 0.33) of this population of molestus would transmit JEV $\geq$16 d after feeding on an animal with a viremia $\geq 10^{4.5}$ PFU/ml. Although this population of molestus was less efficient at transmitting this strain of JEV than were *Culex tritaeniorhynchus* Giles (Okuno et al. 1975, Takahashi 1976), the principal vector of this virus in Asia (Burke and Leake 1988), it was more efficient

at transmitting JEV than were *Culex nigripalpus* Theobald, *Culex quinquefasciatus* Say, and *Culex salinarius* Coquillett from the southeastern United States (M.J.T., unpublished data) or *Cx. quinquefasciatus*, *Aedes vexans* (Meigen), and *Anopheles maculipennis freeborni* Aitken from the western United States (Reeves and Hammon 1946). In contrast, La Motte (1960) reported that *Cx. quinquefasciatus* and both the pipiens and molestus forms of *Cx. pipiens* were highly efficient vectors of JEV. Therefore, it may be necessary to evaluate the ability of specific geographic populations of members of the subgenus *Cx.* (*Culex*) for their ability to transmit selected strains of JEV.

Several authors have speculated on the potential roles of the molestus and pipiens forms of *Cx. p. pipiens* in the transmission of WNV (Savage et al. 1999, Fonseca et al. 2004, Spielman et al. 2004). Specifically, because the pipiens form preferentially feeds on avian hosts, whereas the molestus form are general feeders that will readily feed on humans (Barr 1967), it is likely that the pipiens form is involved primarily as an amplification vector, transmitting WNV from bird to bird, whereas the molestus form may act as a bridge vector, transmitting WNV from the avian cycle to horses and humans. Therefore, areas containing populations of both forms of *Cx. pipiens* may be at greater risk of WNV transmission to mammals. To the best of our knowledge, this is the first study to examine the vector competence of the molestus form of *Cx. pipiens* to transmit WNV. The efficiency with which it transmitted WNV in the laboratory supports the hypothesis that areas where both species are present would be at greater risk of human illness as reported by Savage et al. (1999).

In summary, the molestus form of *Cx. pipiens*, derived from specimens captured near Tashkent, Uzbekistan, were highly efficient laboratory vectors of WNV. Based on this, and the role of *Cx. pipiens* in other geographic locations, this species should be considered to be one of the principal vectors of WNV in Uzbekistan. The molestus form of *Cx. pipiens* was also a moderately competent laboratory vector for JEV and might be able to vector JEV, should that virus be introduced into Uzbekistan. Additional studies need to be conducted to determine feeding preference for this species in Uzbekistan and to evaluate potential differences in vector competence between the molestus form (used in the current study) and the pipiens form of this species for both WNV and JEV.

Acknowledgments

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